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TITLE OF THE INVENTION

NUCLEOTIDE SEQUENCES WHICH CODE FOR THE *oxyR* GENE

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims priority to the US provisional Application No. 60/279,415 filed March 29, 2001, German Application No. DE 10110053.1 filed March 2, 2001, and German Application No. DE 10042052.4 filed August 26, 2000; the entire contents of all three applications are incorporated herein by reference.

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

The invention provides nucleotide sequences from *Coryneform* bacteria which code for the *oxyR* gene and a process for the fermentative preparation of amino acids, in particular L-lysine, using bacteria in which the *oxyR* gene is enhanced. The *oxyR* gene codes for the transcription regulator OxyR, which belongs to the LysR family.

DISCUSSION OF THE BACKGROUND

L-Amino acids, particularly L-lysine, are used in human medicine and in the pharmaceuticals industry, in the foodstuffs industry and, most particularly in animal nutrition.

It is known that amino acids are prepared by fermentation from strains of *Coryneform* bacteria, in particular *Corynebacterium glutamicum*. Because of their great importance, attempts are constantly being made to improve the preparation processes. Improvements to the process may concern measures relating to fermentation, for example, stirring and oxygen supply, or the composition of the nutrient media, such as, the sugar concentration during the fermentation, or the working up to the product form by, for example, ion exchange chromatography, or the intrinsic output properties of the microorganism itself.

The output properties of these microorganisms are improved by employing methods of mutagenesis, selection, and mutant selection. These methods yield strains that produce amino acids, such as the lysine analogue S-(2-aminoethyl)-cysteine, and are resistant to antimetabolites or are auxotrophic for metabolites important for regulation. In this manner, L-lysine can be obtained from these strains.

For a number of years, methods of the recombinant DNA technology have also been used for improving L-amino acid-producing strains of *Corynebacterium*. However, there remains a critical need for improved methods of producing L-amino acids and thus for the provision of strains of bacteria producing higher amounts of L-amino acids. On a commercial or industrial scale even small improvements in the yield of L-amino acids, or the efficiency of their production, are economically significant. Prior to the present invention, it was not recognized that enhanced expression of the *oxyR* gene encoding the OxyR transcriptional regulator would improve L-amino acid yields.

SUMMARY OF THE INVENTION

An object of the present invention is to provide novel measures for the production of amino acids or L-amino acids, where these amino acids include L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine and the salts (monohydrochloride or sulfate) thereof.

One object of the present invention is providing a novel process for improving the fermentative production of said L-amino acids, particularly L-lysine. Such a process includes enhanced bacteria, preferably enhanced *Coryneform* bacteria, which express enhanced amounts of the OxyR transcriptional regulator, which is encoded by *oxyR* gene.

Thus, another object of the present invention is providing such a bacterium, which expresses an enhanced amount of OxyR transcriptional regulator or gene products of the *oxyR* gene.

Another object of the present invention is providing a bacterium, preferably a *Coryneform* bacterium, which expresses a polypeptide that has enhanced OxyR transcriptional regulator activity.

Another object of the invention is to provide a nucleotide sequence encoding a polypeptide which has OxyR transcriptional regulator protein sequence. One embodiment of such a sequence is the nucleotide sequence of SEQ ID NO: 1.

A further object of the invention is a method of making OxyR transcriptional regulator or an isolated polypeptide having a OxyR transcriptional regulator activity, as well as use of such isolated polypeptides in the production of amino acids. One embodiment of such a polypeptide is the polypeptide having the amino acid sequence of SEQ ID NO: 2.

Other objects of the invention include methods of detecting nucleic acid sequences homologous to SEQ ID NO: 1, particularly nucleic acid sequences encoding polypeptides

that have OxyR transcriptional regulator activity, and methods of making nucleic acids encoding such polypeptides.

The above objects highlight certain aspects of the invention. Additional objects, aspects and embodiments of the invention are found in the following detailed description of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Map of the plasmid pEC-T18mob2.

Figure 2: Map of the plasmid pT-oxyRexp.

DETAILED DESCRIPTION OF THE INVENTION

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art of molecular biology. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described herein. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be limiting.

Reference is made to standard textbooks of molecular biology that contain definitions and methods and means for carrying out basic techniques, encompassed by the present invention. See, for example, Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York (1982) and Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York (1989) and the various references cited therein.

The invention provides an isolated polynucleotide from *Coryneform* bacteria, containing a polynucleotide sequence coding for the *oxyR* gene, selected from the group consisting of

a) polynucleotide that is at least 70 % identical to a polynucleotide that codes for a polypeptide containing the amino acid sequence of SEQ ID No. 2,

b) polynucleotide that codes for a polypeptide containing an amino acid sequence that is at least 70 % identical to the amino acid sequence of SEQ ID No.2,

c) polynucleotide that is complementary to the polynucleotides of a) or b), and

d) polynucleotide containing at least 15 successive nucleotides of the polynucleotide sequence of a), b) or c),

the polypeptide preferably having the activity of the transcription regulator OxyR.

The invention also provides the above-mentioned polynucleotide, preferably being a replicable DNA containing:

(i) the nucleotide sequence shown in SEQ ID No.1, or

(ii) at least one sequence that corresponds to sequence (i) within the range of degeneracy of the genetic code, or

(iii) at least one sequence that hybridizes with the sequences that are complementary to sequence (i) or (ii), and optionally

(iv) sense mutations in (i) that are neutral in terms of function.

The invention also provides:

polynucleotides containing at least 15 successive nucleotides chosen from the nucleotide sequence of SEQ ID No. 1 between positions 1 and 490;

polynucleotides containing at least 15 successive nucleotides chosen from the nucleotide sequence of SEQ ID No. 1 between positions 491 and 1471; and

polynucleotides containing at least 15 successive nucleotides chosen from the nucleotide sequence of SEQ ID No. 1 between positions 1472 and 1675.

Additional provisions of this invention are:

a replicable DNA containing the nucleotide sequence as shown in SEQ ID No. 1;

a polynucleotide that codes for a polypeptide containing the amino acid sequence as shown in SEQ ID No. 2;

a vector containing the DNA sequence of *Corynebacterium glutamicum* which codes for the *oxyR* gene, deposited in *Corynebacterium glutamicum* as pT-oxyRexp under DSM 13457 at the DSMZ, Braunschweig (Germany);

and, *Coryneform* bacteria that contain the vector carrying the *oxyR* gene or in which the *oxyR* gene expression is enhanced.

The invention also provides polynucleotides consisting substantially of a polynucleotide sequence, which are obtainable by screening by means of hybridization, of a corresponding *Coryneform* gene library that contains the complete gene having the polynucleotide sequence according to SEQ ID No.1 or parts thereof, using a probe containing the sequence of said polynucleotide according SEQ ID No.1 or a fragment thereof, and isolating said polynucleotide sequence.

Polynucleotide sequences according to the invention are suitable as hybridization probes for RNA, cDNA and DNA, in order to isolate the full length nucleic acids or polynucleotides or genes that code for the transcription regulator OxyR, or in order to isolate nucleic acids or polynucleotides or genes that have a high similarity with the sequence of the *oxyR* gene. These polynucleotide sequences are also suitable for incorporation into arrays, micro-arrays or DNA-chips in order to detect and determine the corresponding polynucleotides.

The DNA of genes that code for the transcription regulator OxyR can be prepared with the polymerase chain reaction (PCR) by using polynucleotides according to the invention as primers.

Such oligonucleotides acting as probes or primers contain at least 25-30, preferably at least 20-24, more preferably at least 15-19 consecutive nucleotides. Also suitable are oligonucleotides that have a length of at least 31- 40 or at least 41- 50 nucleotides. Additional oligonucleotides that are suitable have a length of at least 100, 150, 200, 250 or 300 nucleotides.

"Isolated" means removed out of its natural environment.

"Polynucleotide" generally refers to polyribonucleotides and polydeoxyribonucleotides. The RNA or DNA may be modified or un-modified.

The polynucleotides according to the invention include a polynucleotide shown in SEQ ID No. 1 or a fragment prepared therefrom and also those that are at least 70% to 80%, preferably at least 81% to 85%, more preferably at least 86% to 90%, and most preferably at least 91% to 99% identical to the polynucleotide according to SEQ ID No. 1 or a fragment prepared therefrom.

"Polypeptides" are understood as being peptides or proteins that comprise two or more amino acids bonded via peptide bonds.

The polypeptides according to the invention include a polypeptide shown in SEQ ID No. 2, particularly those with the biological activity of the transcription regulator OxyR, and also those that are at least 70% to 80%, preferably at least 81% to 85%, more preferably at least 86% to 90%, and most preferably at least 91% to 99% identical to the polypeptide according to SEQ ID No. 2 and exhibit the mentioned activity.

The invention also provides a process for the production of amino acids, where these amino acids include L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine using *Coryneform* bacteria that, in particular,

already produce amino acids and in which the nucleotide sequences coding for the *oxyR* gene are enhanced, in particular over-expressed.

The term "enhancement" in this connection describes the increase in the intracellular activity of one or more enzymes in a microorganism that are coded by the corresponding DNA, by, for example increasing the number of copies of the gene or genes, using a potent promoter, or using a gene or allele coding for a corresponding enzyme having a high activity, and optionally combining these measures.

The microorganisms provided by the present invention may prepare L-amino acids, in particular L-lysine, from glucose, sucrose, lactose, fructose, maltose, molasses, starch, cellulose or from glycerol and ethanol. The microorganisms may be representatives of *Coryneform* bacteria, in particular of the genus *Corynebacterium*. *Corynebacterium glutamicum* species of this genus garners special since it is well known to those skilled in the art for its ability to produce L-amino acids.

Suitable strains of the genus *Corynebacterium*, in particular of the species *Corynebacterium glutamicum* (*C. glutamicum*), are, in particular the known wild-type strains

Corynebacterium glutamicum ATCC13032

Corynebacterium acetoglutamicum ATCC15806

Corynebacterium acetoacidophilum ATCC13870

Corynebacterium thermoaminogenes FERM BP-1539

Corynebacterium melassecola ATCC17965

Brevibacterium flavum ATCC14067

Brevibacterium lactofermentum ATCC13869, and

Brevibacterium divaricatum ATCC14020

or L-amino acid-producing mutants or strains prepared therefrom, for example, the L-lysine producing strains

Corynebacterium glutamicum FERM-P 1709

Corynebacterium glutamicum FERM-P 6463

Corynebacterium glutamicum FERM-P 6464

Corynebacterium glutamicum DSM5715.

Brevibacterium flavum FERM-P 1708, and

Brevibacterium lactofermentum FERM-P 1712

Preferably, a bacterial strain with enhanced expression of a *oxyR* gene that encodes a polypeptide with transcription regulator OxyR activity will improve amino acid yield at least 1%.

The inventors have succeeded in isolating the new *oxyR* gene of *C. glutamicum* that codes for the transcription regulator OxyR.

To isolate the *oxyR* gene or also other genes of *C. glutamicum*, a gene library of that microorganism is prepared in *Escherichia coli* (*E. coli*). The preparation of gene libraries is described in generally known textbooks and handbooks. For example, the textbook of Winnacker: Gene und Klone, Eine Einführung in die Gentechnologie (Verlag Chemie, Weinheim, Germany, 1990) or the handbook by Sambrook et al.: Molecular Cloning, A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1989). A well-known gene library is that of the *E. coli* K-12 strain W3110, which has been prepared by Kohara et al. (Cell 50, 495 -508 (1987)) in λ vectors. Bathe et al. (Molecular and General Genetics, 252:255-265, 1996) describe a gene library of *C. glutamicum* ATCC13032, which was prepared with the aid of the cosmid vector SuperCos I (Wahl et al., 1987, Proceedings of the National Academy of Sciences USA, 84:2160-2164) in the *E.coli* K-12 strain NM554 (Raleigh et al., 1988, Nucleic Acids Research 16:1563-1575). Börmann et al. (Molecular Microbiology 6(3), 317-326) (1992)) in turn describe a gene library of *C. glutamicum* ATCC13032 using the cosmid pHC79 (Hohn and Collins, Gene 11, 291-298 (1980)).

It is possible to use plasmids such as pBR322 (Bolivar, Life Sciences, 25, 807-818 (1979)) or pUC9 (Viera et al., 1982, Gene, 19:259-268) in order to prepare a gene library of *C. glutamicum* in *E. coli*. Suitable hosts are particularly those *E. coli* strains, which are restriction- and recombination-deficient, such as the strain DH5αMCR, which has been described by Grant et al. (Proceedings of the National Academy of Sciences USA, 87 (1990) 4645-4649).

The long DNA fragments cloned with the aid of cosmids or other λ vectors can then be subcloned into the usual vectors suitable for sequencing as described *inter alia* by Sanger et al. (Proceedings of the National Academy of Sciences of the United States of America, 74:5463-5467, 1977).

The resulting DNA sequences can then be investigated with known algorithms or sequence analysis programs, such as that of Staden (Nucleic Acids Research 14, 217-232 (1986)),that of Marck (Nucleic Acids Research 16, 1829-1836 (1988)) or the GCG program of Butler (Methods of Biochemical Analysis 39, 74-97 (1998)).

In that manner, the novel DNA sequence of *C. glutamicum* that codes for the *oxyR* gene (SEQ ID No. 1) has been obtained and forms part of this invention. Furthermore, the amino acid sequence of the corresponding protein has been derived from the present DNA

sequence by the methods described above. The resulting amino acid sequence of the *oxyR* gene product is shown in SEQ ID No. 2. It is known to those skilled in the art that enzymes endogenous in the host can remove the N-terminal amino acid methionine or formylmethionine, as such the resulting form of the transcription regulator OxyR forms part of this invention.

Coding DNA sequences that result from SEQ ID No. 1 by the degeneracy of the genetic code also form part of the invention. In the same way, DNA sequences that hybridize with SEQ ID No. 1 or parts of SEQ ID No. 1 form part of the invention. Furthermore, to a person skilled in the art, conservative amino acid exchanges, such as exchange of glycine for alanine or of aspartic acid for glutamic acid in proteins, are known as "sense mutations." These mutations do not lead to a fundamental change in the activity of the protein, i.e. are neutral in terms of function. It is also known that changes on the N and/or C terminus of a protein may not substantially impair or may even stabilize the function thereof. The person skilled in the art will find relevant information *inter alia* in Ben-Bassat et al. (Journal of Bacteriology 169:751-757 (1987)), in O'Regan et al. (Gene 77:237-251 (1989)), in Sahin-Toth et al. (Protein Sciences 3:240-247 (1994)), in Hochuli et al. (Bio/Technology 6:1321-1325 (1988)) and in known textbooks of genetics and molecular biology. Amino acid sequences that result in a corresponding manner from SEQ ID No. 2 also form part of the invention.

DNA sequences that hybridize with SEQ ID No. 1 or parts of SEQ ID No. 1 form part of the invention. Finally, DNA sequences that are prepared by the polymerase chain reaction (PCR) using primers that result from SEQ ID No. 1 form part of the invention. Such oligonucleotides typically have a length of at least 15 nucleotides.

A person skilled in the art will find instructions for identifying DNA sequences by means of hybridization *inter alia* in the handbook "The DIG System Users Guide for Filter Hybridization" from Boehringer Mannheim GmbH (Mannheim, Germany, 1993) and in Liebl et al. (International Journal of Systematic Bacteriology (1991) 41: 255-260). Hybridization takes place under stringent conditions, that is to say only hybrids in which the probe and target sequence, i.e. the polynucleotides treated with the probe, are at least 70 % identical are formed. It is known that the stringency of the hybridization, including the washing steps, is influenced or determined by varying the buffer composition, the temperature, and the salt concentration. For reasons explained *infra*, the hybridization reaction is preferably preferably under a relatively low stringency compared with the washing steps (Hybaid Hybridisation Guide, Hybaid Limited, Teddington, UK, 1996).

A 5x SSC buffer at a temperature of approx. 50 - 68°C, for example, can be employed for the hybridization reaction. Probes can also hybridize here with polynucleotides which are less than 70 % identical to the sequence of the probe. Such hybrids are less stable and are removed by washing under stringent conditions. This can be achieved, for example, by lowering the salt concentration to 2x SSC and subsequently 0.5x SSC (The DIG System User's Guide for Filter Hybridisation, Boehringer Mannheim, Mannheim, Germany, 1995) with a temperature of approximately 50 - 68°C being established. It is optionally possible to lower the salt concentration to 0.1x SSC. Polynucleotide fragments which are, for example, at least 70 % or at least 80 % or at least 90 % to 95 % identical to the sequence of the probe employed can be isolated by increasing the hybridization temperature stepwise in approximately 1 - 2°C increments. Commercial kits containing further instructions on hybridization are readily obtainable (e.g. DIG Easy Hyb from Roche Diagnostics GmbH, Mannheim, Germany, Catalogue No. 1603558).

A person skilled in the art will find instructions for amplification of DNA sequences with the aid of the polymerase chain reaction (PCR) *inter alia* in the handbook by Gait: Oligonucleotide Synthesis: A Practical Approach (IRL Press, Oxford, UK, 1984) and in Newton and Graham: PCR (Spektrum Akademischer Verlag, Heidelberg, Germany, 1994).

During work on the present invention it was found that *Coryneform* bacteria produce amino acids, in particular L-lysine, in an improved manner after over-expression of the *oxyR* gene.

To achieve an over-expression, the number of copies of the corresponding genes may be increased, or the promoter and regulation region, or the ribosome binding site upstream of the structural gene can be mutated. Expression cassettes that are incorporated upstream of the structural gene may also facilitate over-expression. Utilization of inducible promoters may also increase the expression of a desired gene in the course of production of L-lysine by fermentation. The expression is also improved by measures that prolong the life of the m-RNA. Furthermore, preventing the degradation of the enzyme may also increase the enzyme's activity. The genes or gene constructs can either be present in plasmids with a varying number of copies, or can be integrated and amplified in the chromosome. Alternatively, changing the composition of the media and the culture procedure may lead to over-expression of the desired gene.

A person skilled in the art will find instructions in this context *inter alia* in Martin et al. (Bio/Technology 5, 137-146 (1987)), in Guerrero et al. (Gene 138, 35-41 (1994)), Tsuchiya and Morinaga (Bio/Technology 6, 428-430 (1988)), in Eikmanns et al. (Gene 102,

93-98 (1991)), in European Patent Specification 0 472 869, in US Patent 4,601,893, in Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991), in Reinscheid et al. (Applied and Environmental Microbiology 60, 126-132 (1994)), in LaBarre et al. (Journal of Bacteriology 175, 1001-1007 (1993)), in Patent Application WO 96/15246, in Malumbres et al. (Gene 134, 15 – 24 (1993)), in Japanese Laid-Open Specification JP-A-10-229891, in Jensen and Hammer (Biotechnology and Bioengineering 58, 191-195 (1998)), in Makrides (Microbiological Reviews 60:512-538 (1996)) and in known textbooks of genetics and molecular biology.

By way of example, over-expression of the oxyR gene according to this invention was achieved with the aid of episomal plasmids. Suitable plasmids are those that are replicated in *Coryneform* bacteria. Numerous known plasmid vectors, such as pZ1 (Menkel et al., Applied and Environmental Microbiology (1989) 64: 549-554), pEKEx1 (Eikmanns et al., Gene 102:93-98 (1991)) or pHS2-1 (Sonnen et al., Gene 107:69-74 (1991)) are based on the cryptic plasmids pHM1519, pBL1 or pGA1. Other suitable plasmid vectors include those based on pCG4 (US-A 4,489,160) or pNG2 (Serwold-Davis et al., FEMS Microbiology Letters 66, 119-124 (1990)) or pAG1 (US-A 5,158,891).

An example of an *oxyR* over-expression plasmid is the *E.coli-C.glutamicum* shuttle vector pT-oxyRexp (Figure 2). This vector contains the replication region *rep* of the plasmid pGA1 including the replication effector *per* (US-A- 5,175,108; Nesvera et al., Journal of Bacteriology 179, 1525-1532 (1997)), the tetracycline resistance-imparting *tetA(Z)* gene of the plasmid pAG1 (US-A- 5,158,891; gene library entry at the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) with accession number AF121000, the replication origin *oriV* of the plasmid pMB1 (Sutcliffe, Cold Spring Harbor Symposium on Quantitative Biology 43, 77-90 (1979)), the *lacZα* gene fragment including the *lac* promoter and a "multiple cloning site" (mcs) (Norrander, J.M. et al. Gene 26, 101-106 (1983)) and the mob region of the plasmid RP4 (Simon et al.,(1983) Bio/Technology 1:784-791).

Also suitable to achieve over-expression of the *oxyR* gene are plasmid vectors that are integrated into the chromosome, as has been described for duplication or amplification of the hom-thrB operon by Reinscheid et al. (Applied and Environmental Microbiology 60, 126-132 (1994)). In this method, the complete gene is cloned into a plasmid vector that can replicate in a host (typically *E. coli*), but not in *C. glutamicum*. Suitable vectors are, for example, pSUP301 (Simon et al., Bio/Technology 1, 784-791 (1983)), pK18mob or pK19mob (Schäfer

et al., Gene 145, 69-73 (1994)), pGEM-T (Promega Corporation, Madison, WI, USA), pCR2.1-TOPO (Shuman (1994). Journal of Biological Chemistry 269:32678-84; US-A 5,487,993), pCR®Blunt (Invitrogen, Groningen, Holland; Bernard et al., Journal of Molecular Biology, 234: 534-541 (1993)), pEM1 (Schrumpf et al, 1991, Journal of Bacteriology 173:4510-4516) or pBGS8 (Spratt et al.,1986, Gene 41: 337-342). The plasmid vector containing the gene to be amplified is then transferred into the desired strain of *C. glutamicum* by conjugation or transformation. The method of conjugation is described, for example, by Schäfer et al. (Applied and Environmental Microbiology 60, 756-759 (1994)). Methods for transformation are described, for example, by Thierbach et al. (Applied Microbiology and Biotechnology 29, 356-362 (1988)), Dunican and Shivnan (Bio/Technology 7, 1067-1070 (1989)) and Tauch et al. (FEMS Microbiological Letters 123, 343-347 (1994)). After homologous recombination by means of a "cross-over" event, the resulting strain contains at least two copies of the gene in question.

In addition, it may be advantageous for the production of L-amino acids, in particular L-lysine, to enhance one or more enzymes of the particular biosynthesis pathway, of glycolysis, of anaplerosis, of the pentose phosphate cycle, of the citric acid cycle or of amino acid export and optionally regulatory proteins, in addition to the oxyR gene.

Thus, for example, for the preparation of amino acids, in particular L-lysine, one or more genes chosen from the group consisting of

- the *dapA* gene which codes for dihydrodipicolinate synthase (EP-B 0 197 335),
- the *gap* gene which codes for glyceraldehyde 3-phosphate dehydrogenase (Eikmanns (1992). Journal of Bacteriology 174:6076-6086),
- the *tpi* gene which codes for triose phosphate isomerase (Eikmanns (1992). Journal of Bacteriology 174:6076-6086),
- the *pgk* gene which codes for 3-phosphoglycerate kinase (Eikmanns (1992). Journal of Bacteriology 174:6076-6086),
- the *pyc* gene which codes for pyruvate carboxylase (Peters-Wendisch et al.(Microbiology 144, 915 - 927 (1998)),
- the *lysC* gene which codes for a feed back resistant aspartate kinase (EP-B-0387527; EP-A-0699759; WO 00/63388)
- the *lysE* gene which codes for lysine export (DE-A-195 48 222)
- the *mqo* gene which codes for malate-quinone oxidoreductase (Molenaar et al. (1998), European Journal of Biochemistry 254: 395-403),

- the *zwf* gene which codes for glucose 6-phosphate dehydrogenase (JP-A-09224661),
- the *gnd* gene which codes for 6-phosphogluconate dehydrogenase (US: 09/531,265),
- the *sod* gene which codes for superoxide dismutase (US: 09/373,731),
- the *zwa1* gene which codes for the Zwa1 protein (DE: 199 59 328.0, DSM 13115)

may be enhanced, in particular over-expressed, concomitant with *oxyR* gene over-expression.

It may also be advantageous for the production of amino acids, in particular L-lysine, in addition to the enhancement of the *oxyR* gene, at the same time to attenuate one or more genes chosen from the group

- the *pck* gene which codes for phosphoenol pyruvate carboxykinase (DE: 199 50,409.1, DSM 13047),
- the *pgi* gene which codes for glucose 6-phosphate isomerase (US: 09/396,478, DSM 12969),
- the *poxB* gene which codes for pyruvate oxidase (DE: 199 51,975.7, DSM 13114),
- the *zwa2* gene which codes for the Zwa2 protein (DE: 199 59,327.2, DSM 13113)

The term "attenuation" in this connection describes the reduction or exclusion of the intracellular activity of one or more enzymes (proteins) in a microorganism that are coded by the corresponding DNA, by, for example using a weak promoter or using a gene or allele which codes for a corresponding enzyme with a low activity or by inactivating the corresponding gene or enzyme (protein), and optionally combining those measures.

It may also be advantageous for the production of amino acids, in particular L-lysine, in addition to over-expression of the oxyR gene, at the same time to eliminate undesirable side reactions, (Nakayama: "Breeding of Amino Acid Producing Micro-organisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

The microorganisms prepared according to the invention, for the purpose of production of L-amino acids, in particular L-lysine, can be cultured by batch process (continuous or discontinuous), fed batch, or repeated fed batch process. A summary of known culture methods is described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

A suitable culture medium must be used to meet the requirements of the particular strains. Descriptions of culture media for various microorganisms are contained in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981).

Sugars and carbohydrates (e.g., glucose, sucrose, lactose, fructose, maltose, molasses, starch and cellulose), oils and fats (e.g., soya oil, sunflower oil, groundnut oil and coconut fat), fatty acids (e.g., palmitic acid, stearic acid and linoleic acid), alcohols (e.g., glycerol and ethanol), and organic acids (e.g., acetic acid) may be used as the carbon source. These substance may be used individually or as a mixture.

Organic nitrogen-containing compounds (e.g., peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea) or inorganic compounds (e.g., ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate) may be used as the nitrogen source. The sources of nitrogen may be used individually or as a mixture.

The phosphorus source may be phosphoric acid, potassium dihydrogen phosphate, or dipotassium hydrogen phosphate (or the corresponding sodium-containing salts). Furthermore, the culture medium must contain salts of metals (e.g., magnesium sulfate or iron sulfate) that are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, may be used in addition to the above-mentioned substances. Moreover, suitable precursors may be added to the culture medium. The starting substances mentioned may be added to the culture in the form of a single batch, or may be added in a suitable manner during fermentation.

Basic compounds (e.g., sodium hydroxide, potassium hydroxide, ammonia or aqueous ammonia) or acidic compounds (e.g., phosphoric acid or sulfuric acid) may be added in a suitable manner to control the pH. Fatty acid polyglycol esters may be used to control the development of foam. In order to maintain the stability of plasmids, suitable substances having a selective action, such as antibiotics, may be added to the medium. In order to maintain aerobic conditions, oxygen or oxygen-containing gas mixtures, such as air, may be introduced into the culture. The temperature of the culture is normally 20°C to 45°C, and preferably 25°C to 40°C. Fermentation is continued until the maximum of the desired product has formed. This objective is normally reached within 10 hours to 160 hours.

Methods for the determination of L-amino acids are known from the prior art. The analysis may thus be carried out, for example, by ion exchange chromatography with subsequent ninhydrin derivation as described by Spackman et al. (Analytical Chemistry, 30,

(1958), 1190) or by reversed phase HPLC as described by Lindroth et al. (Analytical Chemistry (1979) 51: 1167-1174).

The process according to the invention is used for the production of amino acids, in particular L-lysine, by fermentation.

The isolation of plasmid DNA from *Escherichia coli* and all techniques of restriction, Klenow and alkaline phosphatase treatment were performed as described in Sambrook et al. (Molecular Cloning. A Laboratory Manual (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA). Methods for transformation of *Escherichia coli* and the composition of the usual nutrient media, such as LB or TY medium, are also described in this handbook.

The following microorganisms were deposited at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in accordance with the Budapest Treaty:

- *Corynebacterium glutamicum* DSM5715/pT-oxyRexp as DSM 13457, and
- *Escherichia coli* DH5α/pEC-T18mob2 as DSM 13244.

Having generally described this invention, a further understanding can be obtained by reference to certain specific examples, which are provided herein for purposes of illustration only, and are not intended to be limiting unless otherwise specified.

EXAMPLES

Example 1

Preparation of a genomic cosmid gene library from *Corynebacterium glutamicum* ATCC 13032

Chromosomal DNA from *Corynebacterium glutamicum* ATCC 13032 was isolated as described by Tauch et al. (1995, Plasmid 33:168-179) and partly cleaved with the restriction enzyme *Sau*3AI (Amersham Pharmacia, Freiburg, Germany, Product Description *Sau*3AI, Code no. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, Product Description SAP, Code no. 1758250). The DNA of the cosmid vector SuperCos1 (Wahl et al. (1987) Proceedings of the National Academy of Sciences USA 84:2160-2164), obtained from Stratagene (La Jolla, USA, Product Description SuperCos1 Cosmid Vector Kit, Code no. 251301) was cleaved with the restriction enzyme *Xba*I (Amersham Pharmacia, Freiburg,

Germany, Product Description *Xba*I, Code no. 27-0948-02) and likewise dephosphorylated with shrimp alkaline phosphatase.

The cosmid DNA was then cleaved with the restriction enzyme *Bam*HI (Amersham Pharmacia, Freiburg, Germany, Product Description *Bam*HI, Code no. 27-0868-04). The cosmid DNA so treated was mixed with the treated ATCC13032 DNA and the batch was treated with T4 DNA ligase (Amersham Pharmacia, Freiburg, Germany, Product Description T4-DNA-Ligase, Code no.27-0870-04). The ligation mixture was then packed in phages with the aid of Gigapack II XL Packing Extract (Stratagene, La Jolla, USA, Product Description Gigapack II XL Packing Extract, Code no. 200217).

For infection of the *E. coli* strain NM554 (Raleigh et al. 1988, Nucleic Acid Research 16:1563-1575) the cells were taken up in 10 mM $MgSO_4$ and mixed with an aliquot of the phage suspension. The infection and titering of the cosmid library were carried out as described by Sambrook et al. (1989, Molecular Cloning: A laboratory Manual, Cold Spring Harbor), the cells being plated out on LB agar (Lennox, 1955, Virology, 1:190) with 100 mg/l ampicillin. After incubation overnight at 37°C, recombinant individual clones were selected.

Example 2

Isolation and sequencing of the *oxyR* gene

The cosmid DNA of an individual colony was isolated with the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and partly cleaved with the restriction enzyme *Sau*3AI (Amersham Pharmacia, Freiburg, Germany, Product Description *Sau*3AI, Product No. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, Product Description SAP, Product No. 1758250). After separation by gel electrophoresis, the cosmid fragments in the size range of 1500 to 2000 bp were isolated with the QiaExII Gel Extraction Kit (Product No. 20021, Qiagen, Hilden, Germany).

The DNA of the sequencing vector pZero-1, obtained from Invitrogen (Groningen, Holland, Product Description Zero Background Cloning Kit, Product No. K2500-01), was cleaved with the restriction enzyme *Bam*HI (Amersham Pharmacia, Freiburg, Germany, Product Description *Bam*HI, Product No. 27-0868-04). The ligation of the cosmid fragments in the sequencing vector pZero-1 was carried out as described by Sambrook et al. (1989, Molecular Cloning: A laboratory Manual, Cold Spring Harbor), the DNA mixture being

incubated overnight with T4 ligase (Pharmacia Biotech, Freiburg, Germany). This ligation mixture was then electroporated (Tauch et al. 1994, FEMS Microbiol Letters, 123:343-7) into the *E. coli* strain DH5αMCR (Grant, 1990, Proceedings of the National Academy of Sciences U.S.A., 87:4645-4649) and plated out on LB agar (Lennox, 1955, Virology, 1:190) with 50 mg/l zeocin.

Plasmid preparation of the recombinant clones was carried out with Biorobot 9600 (Product No. 900200, Qiagen, Hilden, Germany). DNA sequencing was administered by the dideoxy chain termination method of Sanger et al. (1977, Proceedings of the National Academy of Sciences U.S.A., 74:5463-5467) with modifications according to Zimmermann et al. (1990, Nucleic Acids Research, 18:1067). The "RR dRhodamin Terminator Cycle Sequencing Kit" from PE Applied Biosystems (Product No. 403044, Weiterstadt, Germany) was used. Separation by gel electrophoresis and analysis of the sequencing reaction were carried out in a "Rotiphoresis NF Acrylamide/Bisacrylamide" Gel (29:1) (Product No. A124.1, Roth, Karlsruhe, Germany) with the "ABI Prism 377" sequencer from PE Applied Biosystems (Weiterstadt, Germany).

The raw sequence data obtained were then processed using the Staden program package (1986, Nucleic Acids Research, 14:217-231) version 97-0. The individual sequences of the pZero1 derivatives were assembled to a continuous contig. The computer-assisted coding region analysis was prepared with the XNIP program (Staden, 1986, Nucleic Acids Research, 14:217-231). Further analyses were carried out with the "BLAST search program" (Altschul et al., 1997, Nucleic Acids Research, 25:3389-3402) against the non-redundant databank of the "National Center for Biotechnology Information" (NCBI, Bethesda, MD, USA).

The resulting nucleotide sequence is shown in SEQ ID No. 1. Analysis of the nucleotide sequence revealed an open reading frame of 981 base pairs, which was designated the *oxyR* gene. The *oxyR* gene codes for a protein of 327 amino acids.

Example 3

Preparation of a shuttle vector pT-oxyRexp for enhancement of the *oxyR* gene in *C. glutamicum*

<3.1> Cloning of the *oxyR* gene

From the *C. glutamicum* ATCC strain 13032, chromosomal DNA was isolated by the method of Eikmanns et al. (Microbiology 140: 1817 -1828 (1994)). On the basis of the sequence of the *oxyR* gene known for *C. glutamicum* from Example 2, the following oligonucleotides were chosen for the polymerase chain reaction.

OxyR (oxy-exp; SEQ ID No.3):

5` GAT CGA GAA TTC AAA GGA AGA TCA GCT TAG 3`

OxyR (oxy R2; SEQ ID No.4):

5` GGA AAA CCT CTA GAA AAA CT 3`

The primers shown were synthesized by ARK Scientific GmbH Biosystems (Darmstadt, Germany) and the PCR reaction was carried out by the standard PCR method of Innis et al. (PCR protocols. A guide to methods and applications, 1990, Academic Press) with Pwo-Polymerase from Roche Diagnostics GmbH (Mannheim, Germany). With the aid of the polymerase chain reaction, a 1.43 kbp DNA fragment containing the *oxyR* gene was isolated. Furthermore, the OxyR (oxy-exp) primer contains the *Eco*RI restriction endonuclease cleavage site sequence and the OxyR (oxy R2) contains the *Xba*I restriction endonuclease cleavage site sequence, underlined in the corresponding nucleotide sequence above.

The approximately 1.43 kb DNA fragment containing the *oxyR* gene was ligated into the pCR®Blunt II vector (Bernard et al., Journal of Molecular Biology, 234: 534-541 (1993)) using the Zero Blunt™ Kit of Invitrogen Corporation (Carlsbad, CA, USA; Catalogue Number K2700-20) and subsequently transformed into the *E. coli* strain Top10 (Grant et al., Proceedings of the National Academy of Sciences USA, 87 (1990) 4645-4649) according to the manufacturer's instructions (Invitrogen Corporation, Carlsbad, CA, USA). Plasmid-carrying cells were selected by plating out the transformation batch on LB agar (Sambrook et al., Molecular cloning: A Laboratory Manual. 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989), which had been supplemented with 25 mg/l kanamycin. Plasmid DNA was isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen (Hilden, Germany) and checked by treatment with the restriction enzyme *Xba*I and *Eco*RI with subsequent agarose gel electrophoresis (0.8 %). The DNA sequence of the amplified DNA fragment was verified by DNA sequencing. Accordingly, the plasmid was called pCR-oxyRexp. The strain was called E. coli Top10 / pCR-oxyRexp.

<3.2> Preparation of the *E. coli – C. glutamicum* shuttle vector pEC-T18mob2

The *E. coli – C. glutamicum* shuttle vector was constructed according to the prior art. The vector contains the replication region *rep* of the plasmid pGA1 including the replication effector *per* (US-A- 5,175,108; Nesvera et al., Journal of Bacteriology 179, 1525-1532 (1997)), the tetracycline resistance-imparting *tetA(Z)* gene of the plasmid pAG1 (US-A- 5,158,891; gene library entry at the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) with the accession number AF121000), the replication region *oriV* of the plasmid pMB1 (Sutcliffe, Cold Spring Harbor Symposium on Quantitative Biology 43, 77-90 (1979)), the *lacZα* gene fragment including the *lac* promoter and a multiple cloning site (mcs) (Norrander, J.M. et al. Gene 26, 101-106 (1983)) and the *mob* region of the plasmid RP4 (Simon et al.,(1983) Bio/Technology 1:784-791). The vector constructed was transformed in the *E. coli* strain DH5α (Hanahan, In: DNA cloning. A Practical Approach. Vol. I. IRL-Press, Oxford, Washington DC, USA). Selection of plasmid-carrying cells was carried out by plating out the transformation batch on LB agar (Sambrook et al., Molecular cloning: a laboratory manual. 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.), which had been supplemented with 5 mg/l tetracycline. Plasmid DNA was isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction with the restriction enzyme *Eco*RI and *Hind*III and subsequent agarose gel electrophoresis (0.8 %). The plasmid was called pEC-T18mob2 and is shown in Figure 1.

<3.3> Cloning of *oxyR* into the *E. coli-C. glutamicum* shuttle vector pEC-T18mob2

The *E. coli – C. glutamicum* shuttle vector pEC-T18mob2 described in Example 3.2 was used as the vector. DNA of this plasmid was cleaved completely with the restriction enzymes *Eco*RI and *Xba*I and then dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, Product Description SAP, Product No. 1758250).

The *oxyR* gene was isolated from the plasmid pCR-oxyRexp described in Example 3.1 by complete cleavage with the enzymes *Eco*RI and *Xba*I. The approximately 1.43 kbp DNA fragment containing the *oxyR* gene was isolated from the agarose gel with the QiaExII Gel Extraction Kit (Product No. 20021, Qiagen, Hilden, Germany).

The oxyR fragment obtained in this manner was mixed with the prepared vector pEC-T18mob2 and the batch was treated with T4 DNA ligase (Amersham Pharmacia, Freiburg, Germany, Product Description T4-DNA-Ligase, Code no. 27-0870-04). The ligation batch was transformed into the *E. coli* strain DH5α (Hanahan, In: DNA cloning. A Practical

Approach. Vol. I. IRL-Press, Oxford, Washington DC, USA). Selection of plasmid-carrying cells was made by plating out the transformation batch on LB agar (Lennox, 1955, Virology, 1:190) with 5 mg/l tetracycline. After incubation overnight at 37°C, recombinant individual clones were selected. Plasmid DNA was isolated from a transformant with the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and cleaved with the restriction enzymes *Eco*RI and *Xba*I to check the plasmid by subsequent agarose gel electrophoresis. The resulting plasmid was called pT-oxyRexp. It is shown in Figure 2.

Example 4

Transformation of the strain DSM5715 with the plasmid pT-oxyRexp

The strain DSM5715 was transformed with the plasmid pT-oxyRexp using the electroporation method described by Liebl et al., (FEMS Microbiology Letters, 53:299-303 (1989)). Selection of the transformants took place on LBHIS agar comprising 18.5 g/l brain-heart infusion broth, 0.5 M sorbitol, 5 g/l Bacto-tryptone, 2.5 g/l Bacto-yeast extract, 5 g/l NaCl and 18 g/l Bacto-agar, which had been supplemented with 5 mg/l tetracycline. Incubation was carried out for 2 days at 33°C.

Plasmid DNA was isolated from a transformant by conventional methods (Peters-Wendisch et al., 1998, Microbiology, 144, 915 -927), cleaved with the restriction endonucleases *Eco*RI and *Xba*I, and the plasmid was checked by subsequent agarose gel electrophoresis. The resulting strain was called DSM5715/pT-oxyRexp.

Example 5

Preparation of L-lysine

The *C. glutamicum* strain DSM5715/pT-oxyRexp obtained in Example 4 was cultured in a nutrient medium suitable for the production of L-lysine by fermenation, and the L-lysine content in the culture supernatant was determined.

To that end, the strain was first incubated on an agar plate with the corresponding antibiotic (brain-heart agar with tetracycline (5 mg/l)) for 24 hours at 33°C. A pre-culture was inoculated (10 ml medium in a 100 ml conical flask). The complete CgIII medium was used as the medium for the pre-culture starting from this agar plate culture.

Cg III Medium

NaCl	2.5 g/l
Bacto-Peptone	10 g/l
Bacto-Yeast extract	10 g/l
Glucose (autoclaved separately)	2 % (w/v)
The pH was brought to pH 7.4	

Tetracycline (5 mg/l) was added to the pre-culture medium. The pre-culture was incubated for 16 hours at 33°C at 240 rpm on a shaker. A main culture was inoculated from this pre-culture such that the initial OD (660 nm) of the main culture was 0.05. MM medium was used for the main culture.

Medium MM

CSL (corn steep liquor)	5 g/l
MOPS (morpholinopropanesulfonic acid)	20 g/l
Glucose (autoclaved separately)	50 g/l
$(NH_4)_2SO_4$	25 g/l
KH_2PO_4	0.1 g/l
$MgSO_4 * 7\ H_2O$	1.0 g/l
$CaCl_2 * 2\ H_2O$	10 mg/l
$FeSO_4 * 7\ H_2O$	10 mg/l
$MnSO_4 * H_2O$	5.0mg/l
Biotin (sterile-filtered)	0.3 mg/l
Thiamine * HCl (sterile-filtered)	0.2 mg/l
L-Leucine (sterile-filtered)	0.1 g/l
$CaCO_3$	25 g/l

The CSL, MOPS, and the salt solution were brought to pH 7 with aqueous ammonia and autoclaved. The sterile substrate and vitamin solutions were then added, as well as the dry, autoclaved $CaCO_3$.

Cell-growth was performed in a 10 ml volume in a 100 ml conical flask with baffles. Tetracycline (5 mg/l) was added. Culturing was carried out at 33°C and 80 % atmospheric humidity.

After 72 hours, the OD was determined at a measurement wavelength of 660 nm with a Biomek 1000 (Beckmann Instruments GmbH, Munich). The amount of L-lysine formed was determined with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column derivation with ninhydrin detection.

The result of the experiment is shown in table 1.

Table 1

Strain	OD (660 nm)	Lysine HCl (g/l)
DSM5715	6.8	13.68
DSM5715/pT-oxyRexp	6.5	14.73

Obviously, numerous modifications and variations on the present invention are possible in light of the above teachings. It is therefore to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described herein.